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PRINCIPAL INVESTIGATOR: ZhaoYi Wang, Ph.D.
GuanCheng Li, M.D., Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

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6. AUTHOR(S)
ZhaoYi Wang, Ph.D.
GuanCheng Li, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-Mail: Zwang2@caregroup.harvard.edu

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

We have investigated the ability of Rb associated protein 46 (RbAp46) to inhibit malignant phenotypes of human breast cancer cells in tumorigenesis assays in the past year. We have found that high levels of RbAp46 expression strongly suppressed clonal growth of breast cancer cells in soft-agar and inhibited tumor growth of these cells in nude mice. We have also found that the expression levels of β-catenin were dramatically downregulated in the RbAp46-expressing cells compared with control cells. Concomitantly, the β-catenin/TCF signaling pathway was downregulated and β-catenin was dramatically phosphorylated by GSK-3β at Ser33/37/Thr41 residues in the RbAp46-expressing cells.

The results suggest that high levels of RbAp46 expression inhibit the malignant phenotypes of breast cancer cells through interfering with β-catenin/TCF signaling pathway.

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INTRODUCTION:

Our research program is focused on the study of Rb associated protein 46 (RbAp46), a novel tumor suppressor gene we recently identified and cloned. Long term goal of this study is to understand the role of RbAp46 in abnormal growth of mammary epithelial cells, in particular, the effects of constitutive and high levels of RbAp46 expression on the early development of breast cancer. In the past year (months 13-24), we have finished in vivo xenograft experiments proposed for months 1-12. Furthermore, we have established stable cell lines that express exogenous RbAp46 from human breast cancer cells, MCF7, MDA-MB-231 and MDA-MB-436, and found that high levels of RbAp46 expression strongly inhibit malignant features of these breast cancer cells. We have also found that expression levels of β-catenin, an important signaling molecule, were downregulated in RbAp46-transfected cells compared with control cells. The lower levels of β-catenin were restored by treatment of cells with proteasome inhibitors. We further discovered that the levels of GSK-3β expression were upregulated and β-catenin/Tcf–mediated transcription pathway was strongly downregulated in RbAp46-transfected cells.

BODY:

Task 1: Determining effects of constitutive expression of RbAp46 on the progression of human proliferative breast disease in the MCF10AT xenograft model (Months 1-12).

As described in the annual report of last year, we have established stable MCF10AT3B cell lines expressing exogenous RbAp46. The MCF10AT3B cells are epithelial cells derived from MCF10A cells, a cell line originated from a spontaneous immortalization of non-malignant human mammary gland (Miller *et. al.*, 1993). MCF10A cells transfected with T24 Ha-ras mutant (designated as MCF10AT) acquired the ability to grow as xenografts in the dorsal flank of nude mice. MCF10AT3B cell line was established from a third generation transplant. When xenografted into nude mice, MCF10AT3B cells form atypical hyperplasia, carcinoma in situ and invasive carcinoma (Dawson *et. al.* 1996). This is a widely used and well-characterized model for early development of breast cancer that is suitable for in vitro and in vivo assays.

As described in the last year's report, we have found that (1) overexpression of RbAp46 inhibits abnormal cell growth of MCF10AT3B cells; (2) overexpression of RbAp46 sensitizes MCF10AT3B cells to apoptosis induced by serum starvation through consistent activation of JNK/SAPK pathway; (3) overexpression of RbAp46 induces epithelial-mesenchymal transition in MCF10AT3B epithelial cells.

However, experiments to determine the effects of RbAp46 on the tumorigenicity of MCF10AT3B cells in nude mice as scheduled in months 1-12 (see Approved Statement of Work) were not finished when we prepared the annual report in last year. Now, these experiments have been finished and are described here.

In vivo xenograft experiments were performed (From Approved Statement of Work for months 1-12).

To examine the effect of RbAp46 expression on tumorigenicity of MCF10AT3B cells in nude mice, two established cell lines from each expression vector were injected in the mammary fatpad of 12 nude mice. Forty weeks after injection, tumor like nodules were detected at about 50% of the injected sites of parental cells and control cells transfected with empty vector. When the host animals were sacrificed and the tumor like lesions resected, a portion of each was prepared for histological examination. All lesions showed distended ducts filled with uniform cells containing enlarged nuclei, relatively abundant cytoplasm and sharp cell boundaries (data not shown), corresponding to a grade 4 lesion of proliferative breast disease, i.e. carcinoma in situ (Dawson *et. al.* 1996). However, tumors were totally absent in the mice injected with RbAp46-transfected cells (**Table 1**). This data indicates that RbAp46 strongly suppresses the tumorigenic ability of neoplastigenic MCF10AT3B cells in nude mice.

Task 2: Determining the effects of RbAp46 on transformed phenotypes of breast cancer cells (Months 13-24).

4. Determining if RbAp46 inhibits transformed phenotypes of breast cancer cell lines such as anchorage-independent growth and tumor formation in nude mice. Establishment of Tet-inducible retrovirus system (From Approved Statement of Work).

(1). Constitutive expression of exogenous RbAp46 inhibits transformed phenotypes of breast cancer cells.

We used three human breast cancer cell lines, MDA-MB-231, MDA-MB-436 and MCF7 as tumor models to study the effect of RbAp46 expression on transformed phenotypes of human breast cancer cells. These cell lines are widely used, well-characterized and highly malignant tumor models that are suitable for in vitro and in vivo assays of transformation. We transfected these cells with a CMV promoter-driven expression vector containing HA-tagged RbAp46 and selected transfectants with G418 for three weeks. We established a number of cell lines that expressed exogenous RbAp46 and three of each transfectant are described in detail in this report. We also generated three control cell lines transfected with the empty expression vector for each breast cancer cell line. The clonally isolated colonies were confirmed by Northern blot analysis and Western blot analysis to highly express exogenous RbAp46 (data not shown).

The RbAp46-expressing cells and vector-control cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. The growth rate of each cell line was determined by counting the number of cells daily. The cell lines expressing high levels of exogenous RbAp46 had a two-fold lower growth rate than vector-control cells (data not shown), indicating that the high levels of RbAp46 expression reduce growth rate of transfected breast cancer cells.

We then tested the effects of RbAp46 on transformed phenotypes of these breast cancer cells using two types of experiments. First, we determined the ability of these established cell lines that constitutively express RbAp46 to form colonies in soft-agar. Two hundred cells of each established cell lines were seeded in 60-mm dishes in replicates of five plates for each cell line. After incubation for 3 weeks, colonies with more than 30 cells were counted and scored as anchorage-independent. The RbAp46-expressing cells formed 10-15% fewer colonies in soft-agar than vector-control cells transfected with the empty vector (**Table 2**). Analysis of the data using a student's t-test showed that the reduction in the number of RbAp46-expressing colonies compared with control cells was statistically significant ($p < 0.05$). In addition to the decreased number of colonies, we noted a significant decrease in the average size of colonies formed from cell lines expressing high levels of RbAp46 (data not shown). These results indicate that high levels of RbAp46 expression inhibit the ability of these breast cancer cells to grow anchorage-independently in soft-agar.

Second, we examined the effect of RbAp46 on tumorigenicity of the three breast cancer cell lines in nude mice. Each established cell lines were injected subcutaneously into three nude mice (at two sites each, using 2×10^6 cells per site). Fourteen days after injection, tumors were detected at most sites injected with vector-control cells. However, tumor formation were significantly decreased both in number and in sizes in the mice injected with RbAp46-expressing cells, even after a prolonged period of time (70 days) (**Table 3 and Fig. 1**). Taken together, these data indicated that RbAp46 strongly suppresses the tumorigenic phenotypes of MDA-MB-231, MDA-MB-436 and MCF7 breast cancer cells.

(2). RbAp46 facilitates GSK-dependent protein degradation of β-catenin.

To elucidate the molecular mechanism by which RbAp46 inhibits transformed phenotypes of breast cancer cells, we examined several signaling pathways in RbAp46-transfected MCF7 cells. We found that the expression levels of β-catenin protein were dramatically downregulated in the RbAp46-transfected cells as demonstrated by Western blot analysis whereas no significant change was observed for the levels of its mRNA (**Fig. 2**), suggesting that the steady state levels of β-catenin protein was decreased in RbAp46-transfected cells.

β-catenin is an important regulator of Wnt signal transduction cascade. In absence of Wnt signaling, cytoplasmic β-catenin is associated with APC-Axin/conductin-GSK-3β complex and normally rapidly degraded through proteasome pathway after being phosphorylated by GSK-3β at serine/threonine residues.

It is well established that the peptide aldehyde ALLN (*N*-acetyl-Leu-Leunorleucinal) and MG132 inhibit proteasome-mediated proteolysis, which leads to an accumulation of proteins that are usually metabolized by this pathway (Coux *et al.*, 1996). Upon treatment with ALLN and MG132, the protein levels of β-catenin in RbAp46-transfected cells were increased to a level similar to that of control cells, and the cells which were treated by ALLN clearly showed higher molecular weight forms of β-catenin (**Fig. 3**). These data strongly suggest that the β-catenin protein was destabilized in RbAp46-transfected cells.

To further examine if GSK-3β mediated β-catenin degradation was upregulated in RbAp46-transfected cells, we further determined the expression levels of GSK-3 in RbAp46-transfected cells. We found that the protein levels of both GSK-3α and 3β were dramatically upregulated in RbAp46-transfected MCF7 cells compared with the control cells (**Fig. 4**). Concomitantly, β-catenin was significantly phosphorylated at Ser31/37/Thr41 residues, but not at Thr41/Ser45 residues (data not shown). These results indicated that RbAp46 may upregulate the GSK-3-dependant ubiquitination and proteolysis pathway by which the steady state levels of β-catenin are regulated.

Activation of the Wnt cascade results in an inactivation of GSK-3β and an accumulation of cytosolic β-catenin, which then translocates to the nucleus, binds to transcription factors of the Lef/Tcf family, and activates the transcription of a variety of target genes including c-myc and cyclin D. To confirm β-catenin was functionally downregulated in RbAp46-transfected MCF7cells, Lef/Tcf-dependant nuclear signaling was measured using the TOP-FLASH reporter construct that contains the Lef/Tcf binding site. As a negative control, the FOP-FLASH reporter vector, in which the Lef/Tcf binding sites have been mutated, was also used. The results showed that β-catenin-Lef/Tcf signaling is significantly inhibited in RbAp46-transfected MCF7 cells compared with the control cells (**Fig. 5**).

(3). Establishment of Tet-inducible retrovirus system.

In my original grant proposal, I proposed to set up a Tet-inducible retrovirus system as an alternative approach if we fail to establish stable cell lines expressing exogenous RbAp46.

In the past year, we successfully established a number of stable cell lines to express high levels of exogenous RbAp46 from different breast cancer cells. However, in order to test if induced RbAp46 expression makes tumors to regress, we established a tetracycline-regulated expression system of RbAp46 in MCF7 cells. Western blot analyses showed tightly regulated induction of RbAp46 expression (**Fig. 6A**). No expression of the HA-tagged RbAp46 was observed in the presence of tetracycline by Western blot analysis. Withdrawal of tetracycline led to induction of exogenous RbAp46 expression, detectable within 5 hours. The maximal induction of the transfected RbAp46 was about 27-fold.

To determine the effect of induced RbAp46 expression on growth of MCF7 cells, the cells were seeded at 1 X 10^4 cells/well and counted every other day after tetracycline withdrawal. Induction of RbAp46 resulted in a normal growth rate for about 2 days, followed by a slower growth rate (~2 fold) compared with the control cells (**Fig. 6B**). This data strongly suggested that induced expression of RbAp46 inhibits growth of MCF7 breast cancer cells.

5. Tumor formation assay of Tet-inducible cell lines in nude mice will be performed (From Approved Statement of Work).

As an alternative approach, MCF7 cells carrying Tet-inducible recombinant viruses of RbAp46 have been injected into nude mice. Now, we are still waiting for the appearance of tumors. At various times after tumor appearance, mice with different sized tumors will be provided with water containing no tetracycline to induce the expression of Tet-controlled RbAp46 in the infected cells. The mice will be examined weekly and the size of the tumors in these mice will be compared with the size of tumors in mice given water containing 100 μg/ml of tetracycline. We hope that this experiment will determine whether induction of RbAp46 expression makes tumors regress after they have already formed, and at what stage of progression, RbAp46 can effectively produce this regression.

KEY RESEARCH ACCOMPLISHMENTS:

(1). We have found that overexpression of RbAp46 inhibits transformed phenotypes of breast cancer cells.

(2). We have found that signaling protein β-catenin was destabilized through hyper-phosphorylation by GSK-3β and β-catenin/Tcf–mediated signaling pathway was strongly downregulated in the RbAp46-transfected MCF7 cells.

REPORTABLE OUTCOMES:

1. Li, G-C., Guan, L-S. and **Wang, Z-Y.** "Overexpression of RbAp46 Facilitates Stress-Induced Apoptosis and Suppresses Tumorigenicity of Neoplastigenic Breast epithelial cells" *Submitted.*

2. Li, G-C., Guan, L-S. and **Wang, Z-Y.** "Epithelial-Mesenchymal Transition Induced by RbAp46 is Associated with Destabilization of β-Catenin" *Submitted.*

3. Li, G-C., Guan, L-S., Chen G-C. and **Wang, Z-Y.** "Rb-Associated Protein 46 (RbAp46) Inhibits Cell Growth, Facilitates Stress-Induced Apoptosis and Induces Epithelial-Mesenchymal Transition in Neoplastigenic Mammary Gland Epithelial Cells" *Proceedings Supplement of the American Association for Cancer Research. 2002; 76: 726.*

CONCLUSIONS:

In the past year, significant progress towards the proposed tasks has been made. Experimental results from these progresses are essential for next year study.

We have finished the tumorigenicity assay of MCF10AT3B, a cell line derived from a model of human proliferative breast disease in nude mice and found that constitutive and high levels of RbAp46 expression potently inhibit tumorigenicity of MCF10AT3B cells in nude mice. These results, together with the results reported last year, established that RbAp46, a novel growth inhibitory protein, has potent tumor suppressor activity and may plays an important role in the regulation of normal growth of breast

epithelial cells. Dysregulation of this important protein may contribute to early development of human breast cancer. To our knowledge, this is the first growth inhibitor identified to have tumor suppressor effect on malignant progression of human proliferative breast disease.

Furthermore, we have successfully established stable cell lines from malignant breast cancer cells to constitutively express exogenous RbAp46. These stable cell lines provide valuable tools for future study of RbAp46 function in breast cancer. These cell lines are also valuable assets for research community to study molecular and cellular events during progression of malignant breast cancer. We also established a MCF7 cell line that expresses a tetracycline-inducible RbAp46. This cell line can be used to provide further information about function of RbAp46 in breast cancer and to confirm results that were obtained from the stable cell lines.

We have found that RbAp46 strongly inhibits transformed phenotypes of breast cancer cells. These results suggest that the RbAp46 not only inhibits progression of human proliferative breast disease but also suppresses malignant features of human breast cancer. These data thus further established that RbAp46 is an important player in the regulation of normal growth of mammary epithelial cells, and dysregulation of this protein may lead to tumorigenesis of human breast cancer.

To explore the molecular mechanism by which RbAp46 inhibits transformed phenotypes of breast cancer cells, we examined several signal transduction pathways in RbAp46-transfected MCF7 cells. We found that signaling protein β-catenin was destabilized through hyper-phosphorylation by GSK-3β and β-catenin/Tcf–mediated transcription pathway was strongly downregulated in RbAp46-transfected MCF7 cells.

β-catenin is an important regulator of oncogene Wnt signaling cascade. Cytoplasmic β-catenin levels are important, because dysregulation of β-catenin accumulation as a result of mutations in APC protein and β-catenin itself, which leads to constitutive activation of Wnt-1/β-catenin/Tcf -mediated transcription, has been found to associate with a variety of human cancers. Our finding that β-catenin protein was destabilized in RbAp46-transfected MCF7 cells suggested that RbAp46 may inhibit tumorigencity of breast cancer through downregulation of Wnt-1/β-catenin/Tcf signal transduction pathway. These results thus provided a direction for future study of RbAp46 in early development of human breast cancer. These results also provided new information on the regulation of Wnt-1/β-catenin/Tcf signal transduction pathway during tumorigenesis of a variety of human cancers. Further study of the regulation of Wnt-1/β-catenin/Tcf signal transduction pathway by RbAp46 will not only greatly advance our understanding of human proliferative disease progression but will also lay the foundation for novel approaches to interfere with early develoment of breast cancer.

During the next year of funding, it is planned to further pursue the work proposed in my Approved Statement of Work.

Task 3. Determining the effects of constitutive-expression of RbAp46 on estrogen-stimulated progression of proliferative breast disease. (Months 25-36).

6. Determining the effects of constitutive expression of RbAp46 on growth characteristics of estrogen-stimulated MCF10AT3B cells.

7. Determining the effects of constitutive-expression of RbAp46 on estrogen-stimulated progression of proliferative breast disease in vivo. Thirty-four female nude mice will be used.

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Miller, F.R. Soule, H.D. Tait, L. Pauley, R.J. Wolman, S.R. Dawson, P.J. and Heppner, G.H. (1993). *J. Natl. Cancer. Inst.* 85:1725-1732.

TABLE 1. TUMORIGENICITY OF RbAp46-EXPRESSING MCF10AT3B CELLS IN NUDE MICE

Cell	Tumor
MCF10AT3B	6/12
Vector-2	6/12
Vector-6	7/12
RbAp46-4	0/12
RbAp46-9	0/12

TABLE 2. SOFT-AGAR COLONY FORMATION OF RbAp46-EXPRESSING BREAST CANCER CELLS

MDA-MB-231	colonies	MDA-MB-436	colonies	MCF-7	colonies
Vector (1)	66± 3.3	Vector (3)	78± 4.3	Vector (2)	112± 1.3
Vector (4)	108± 4.1	Vector (4)	84± 5.6	Vector (6)	118± 2.6
Vector (7)	84± 2.4	Vector (6)	69± 3.7	Vector (7)	110± 1.7
RbAp46 (5)	8± 0.8	RbAp46 (7)	23± 1.8	RbAp46 (1)	8.0± 1.8
RbAp46 (9)	8± 0.8	RbAp46 (9)	19± 1.5	RbAp46 (9)	4.0± 0.5
RbAp46 (11)	14± 1.1	RbAp46 (12)	20± 2.2	RbAp46 (10)	12.0± 1.2

TABLE 3. TUMORIGENICITY OF RbAp46-EXPRESSING BREAST CANCER CELLS IN NUDE MICE

MDA-MB-231	Tumor formed	MDA-MB-436	Tumor formed	MCF7	Tumor formed
Vector (1)	6/6	Vector (3)	5/6	Vector (2)	4/6
Vector (4)	4/6	Vector (4)	4/6	Vector (6)	4/6
Vector (7)	3/6	Vector (6)	6/6	Vector (7)	6/6
RbAp46 (5)	0/6	RbAp46 (7)	1/6	RbAp46 (1)	2/6
RbAp46 (9)	0/6	RbAp46 (9)	3/6	RbAp46 (9)	3/6
RbAp46 (11)	0/6	RbAp46 (12)	2/6	RbAp46 (10)	2/6

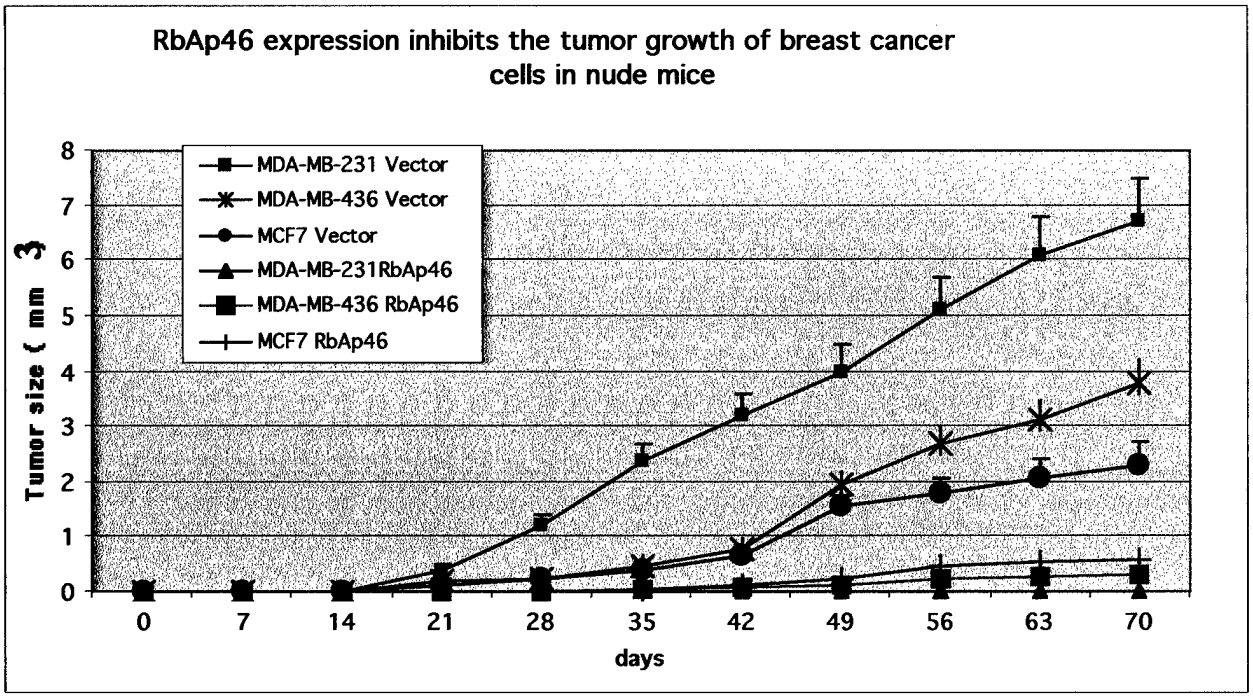

Fig.1 RbAp46-expression inhibits the tumor growth of breast cancer cells in nude mice. Each established cell lines were injected subcutaneously into three nude mice (at two sites each, using 2 X 10^6 cells per site). Tumor size was measured weekly after two weeks.

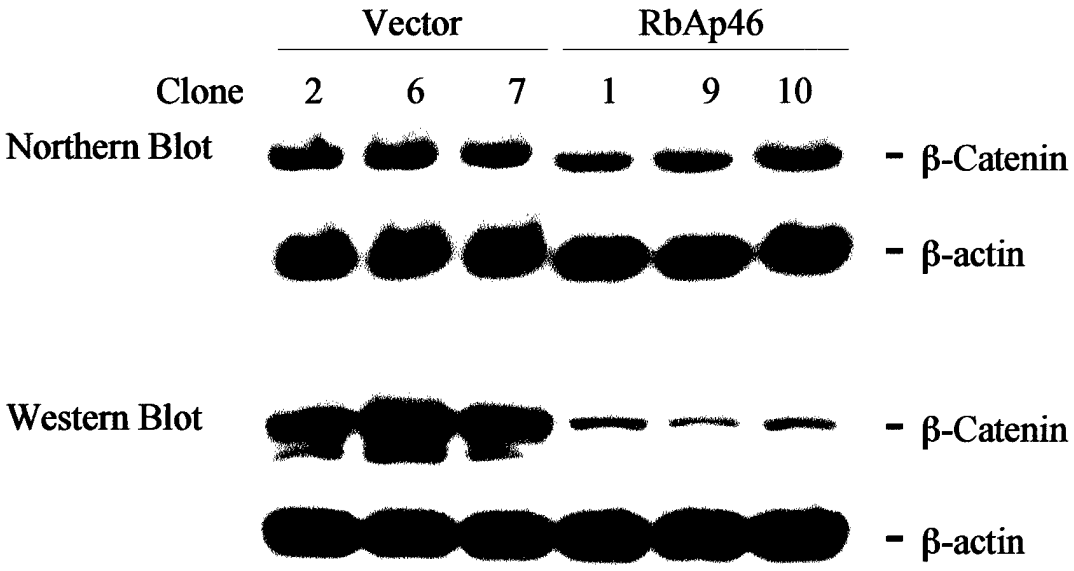

Fig. 2. The protein levels of β-Catenin is downregulated in RbAp46 transfected MCF7 cells. The different clones of RbAp46-transfected MCF7 cells and empty vector transfected cells were cultured under normal condition. Western blot analysis and Northern blot analysis were performed with β-actin as loading controls.

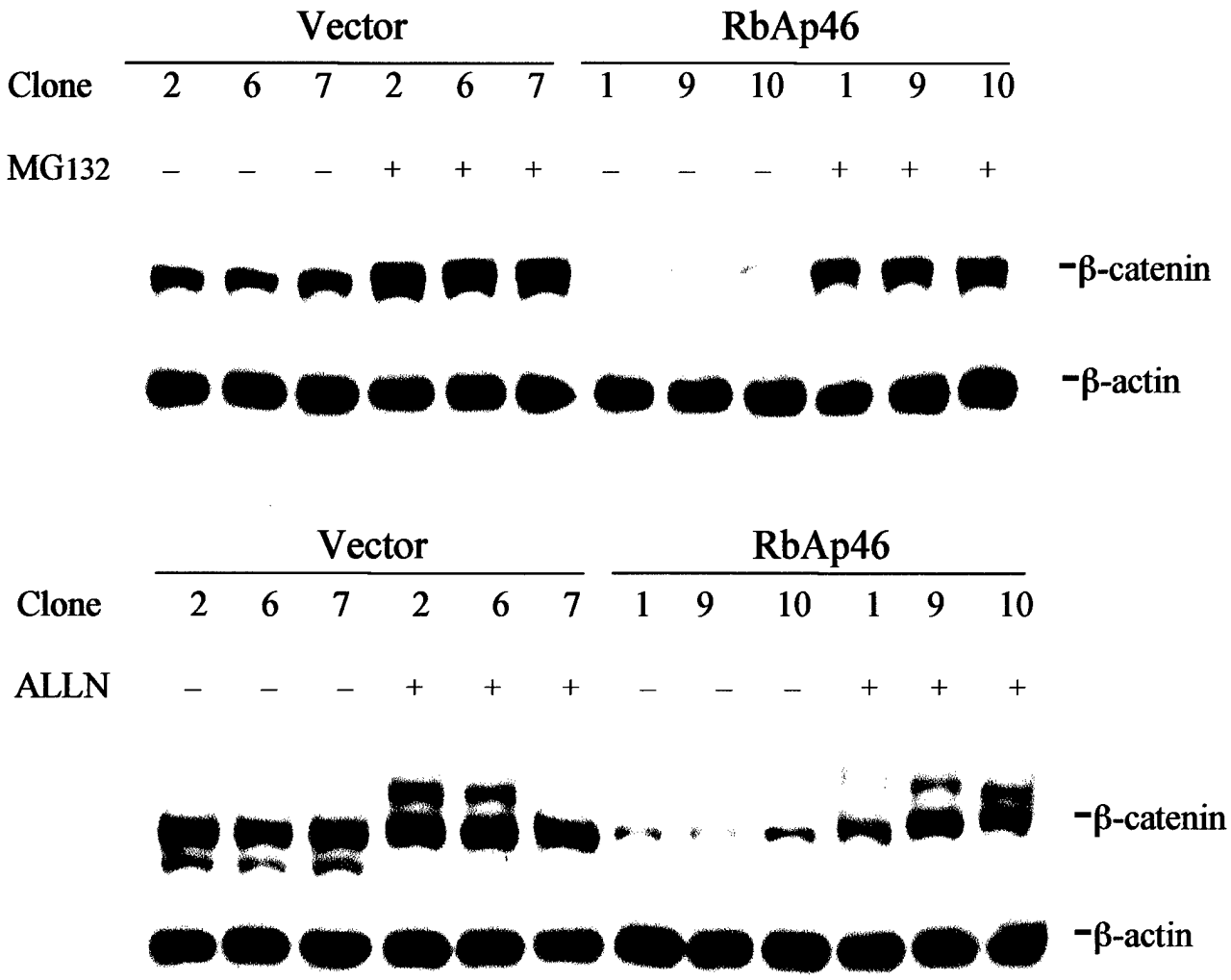

Fig 3. Treatment of RbAp46-transfected MCF7 cells with proteasome inhibitors ALLN and MG132 restores the protein levels of β-catenin. The different clones of RbAp46-transfected MCF7 cells and empty vector transfected cells were cultured under normal condition. The control cells and RbAp46-transfected MCF7 cells were seeded in 6-well plates and treated with proteasome inhibitors, MG-132 and ALLN. The cells were lysed with RIPA lysis buffer. Samples were adjusted to 40 μg of total proteins, and β-catenin levels were analyzed by Western blot analysis with β-actin as loading controls.

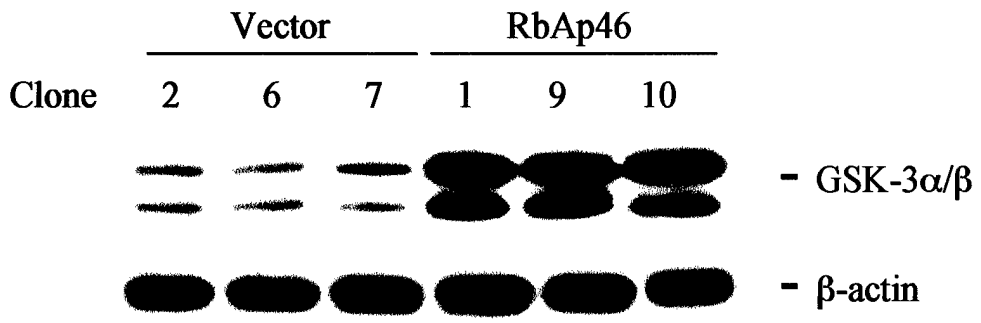

Fig 4. The protein levels of GSK-3α/β are upregulated in RbAp46-transfected MCF7 cells. The cells were lysed and samples were adjusted to 40 μg of total proteins. GSK-3α/β levels were analyzed by SDS-PAGE and Western blot analysis.

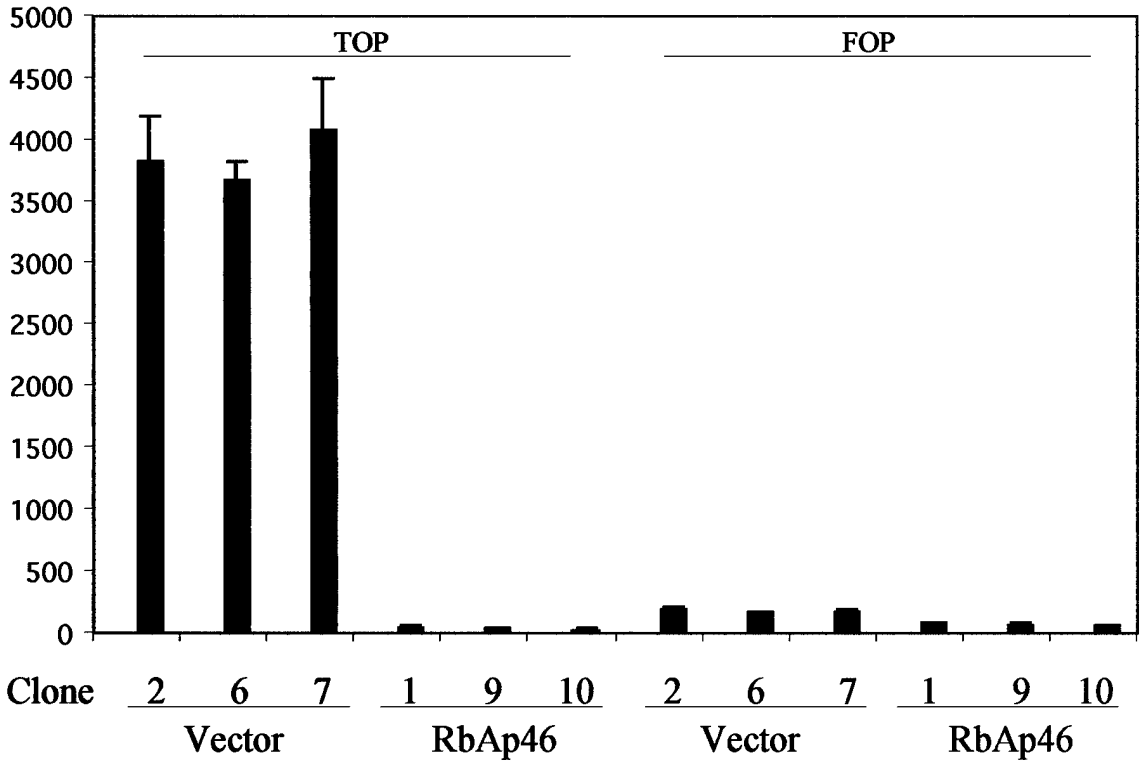

Fig 5. β-catenin-Lef/TCF signaling is downregulated in RbAp46-transfected MCF7 cells. Cells were transfected with Top and Fop reporter plasmids, and pCMV-β-gal plasmid as a transfection control. Forty-eight hours after transfection, luciferase activity was measured and normalized to β-galactosidase activity. All of the data represent more than three independent transfections.

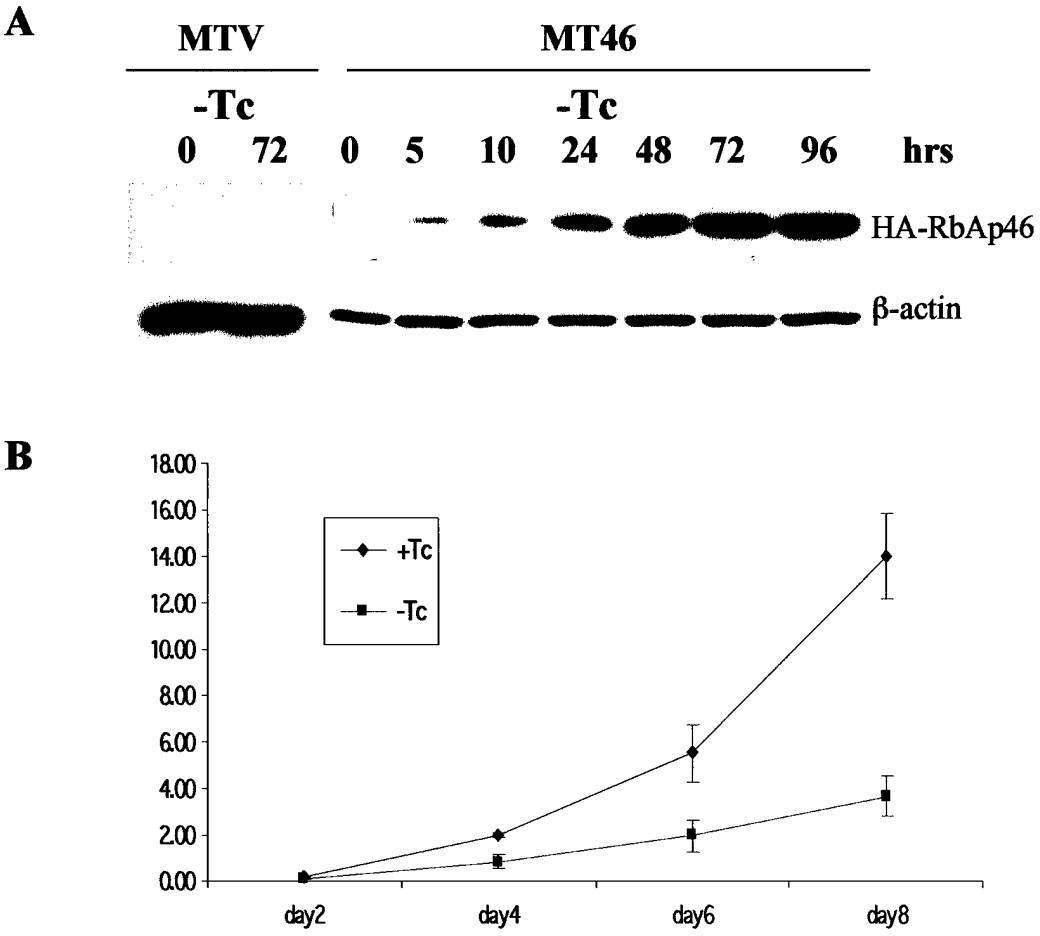

Fig. 6. Inducible expression of RbAp46 inhibits MCF7 cell growth. A. Inducible expression of RbAp46 in MCF7 cells. Western blot analysis of protein extracts prepared from cells infected with RbAp46 containing retrovirus (MT46) and cells infected with empty retrovirus (MTV) growing in the presence (+Tc) or absence (-Tc) of tetracycline for time periods indicated. Anti-HA tag antibody was used to probe the inducible expression of the HA-tagged RbAp46, showing a tightly regulated inducible expression of RbAp46. β-actin was also probed as a loading control. B. Reduction of the growth rate of MT46 cells in the absence of tetracycline. The cells were seeded at 1 X 10^4 cells/well and counted daily in the absence of tetracycline. Data shown represent the mean of three experiments with standard deviations.